NADH CHANNELING AND BINDING OF MALATE DEHYDROGENASE TO BEEF HEART SUBMITOCHONDRIAL PARTICLES

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To My Wife

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I dedicate this thesis to my wife, Julia, for her love.

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NOMENCLATURE[a]

AAT	Aspartate aminotransferase
α-GPD	α-Glycerophosphate dehydrogenase
BCKD	Branched chain keto acid dehydrogenase complex
CPS	Carbamoyl phosphate synthetase
CS	Citrate synthase
GDH	Glutamate dehydrogenase
ICD	Isocitrate dehydrogenase
KGDC	Ketoglutarate dehydrogenase complex
LDH	Lactate dehydrogenase
MDH	Malate dehydrogenase
OTC	Ornithine transcarbamylase
PDC	Pyruvate dehydrogenase complex
PEG	Polyethylene glycol
SMP	Submitochondrial particles
STK	Succinate thiokinase

[a] All other abbreviations are those listed as standard abbreviations not requiring definition by the J. Biol. Chem.

CHAPTER I

INTRODUCTION

Mitochondria

The mitochondrion is the site of eukaryotic oxidative metabolism, in which the Krebs tricarboxylic acid cycle and most of the associated reactions occur. There are two separate membranes in mitochondria, a smooth outer membrane and an elaborately folded inner membrane. The inner membrane separates the mitochondrion into two distinct spaces, the internal or matrix space and the intermembrane space, between the inner and outer membranes. The inner and outer membranes of mitochondria have very different properties. The outer membrane has only a few known enzymatic activities, and is permeable to molecules with molecular weights of up to about 5,000 Dalton. The inner membrane has an unusually high ratio of protein to phospholipid, and is impermeable to most ions and polar molecules. This permeability barrier prevents protons or cofactors such as NADH from moving freely between the mitochondrial matrix and the intermembrane space. The proteins associated with the inner membrane include enzyme systems that are responsible for oxygen consumption and the formation of ATP. Four respiratory complexes of the electron-transport chain, NADH-ubiquinone oxidoreductase (Complex I), Succinate-ubiquinone oxidoreductase (Complex II), Ubiquinone-cytochrome c oxidoreductase (Complex III) and Cytochrome oxidase (Complex IV), are embedded in the inner mitochondrial membrane. Two of the enzymes in the Krebs tricarboxylic acid cycle, succinate dehydrogenase (EC 1.3.99.1) and the α-ketoglutarate dehydrogenase complex (EC 2.3.1.61, 1.6.4.3, 1.2.4.1) are also associated with the inner membrane. The other enzymes of the Krebs cycle, citrate synthase (EC 4.1.3.7), aconitase

(EC 4.2.1.3), NAD^+-isocitrate dehydrogenase (EC 1.11.1.41), succinyl-CoA synthase (EC 6.2.1.4), fumarase (EC 4.2.1.2) and malate dehydrogenase (EC 1.1.1.37) are found in the matrix [1]. In addition to the enzymes of the Krebs cycle, the mitochondrial matrix also contains the enzymes for converting fatty acids, amino acids, and carbohydrates to the Krebs cycle intermediates, as well as substrates, nucleotide cofactors, inorganic ions and the mitochondrial genetic machinery - DNA, RNA, and ribosomes. Thus the matrix is a gel-like substance of < 50% water. Enzymes of the mitochondrial matrix account for about 60% of the total protein of the mitochondrion. Sonication of the inner mitochondrial membrane yields sealed, inside-out vesicles, termed submitochondrial particles (SMP).

Complex I

The electron-transport chain consists of four protein complexes through which electrons pass from lower to higher standard reduction potentials (Figure 1). Electrons are carried from Complex I and Complex II to Complex III and from Complex III to Complex IV. The enzyme system from Complex I via Complex III to Complex IV is called "NADH oxidase". Antimycin A is a specific inhibitor to block electron transfer between ubiquinone and cytochrome c at the reaction site. Thus the activity of "NADH oxidase" (Complex I + Complex III + Complex IV) can be inhibited by antimycin A.

Complex I, which oxidizes NADH and transfers 2 electrons to ubiquinone, is the largest and most complicated of the mitochondrial electron-transfer complexes. It appears to consist of approximately 26 different subunits [2], with a total molecular weight of 850 kDa. The functions of most of these components are unknown. Extensive chemical labelling, cross-linking, and immunological studies have identified the subunits that are exposed on either side of the mitochondrial inner membrane [2] and suggested the positional relationship of many of the subunits. About 16 subunits comprise a hydrophobic sheath within which two water soluble fractions reside. All nine subunits comprising the two water soluble fractions of Complex I are exposed on the matrix side of

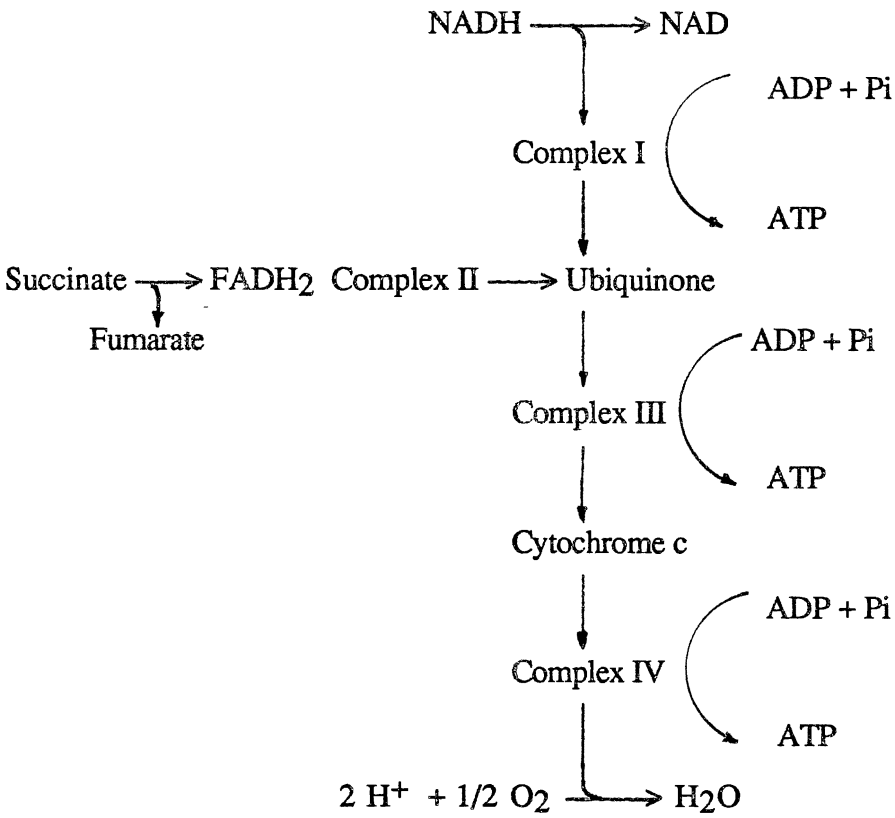


Figure 1. The Mitochondrial Electron-Transport Chain

the inner mitochondrial membrane. In addition to the NADH dehydrogenase activity, Complex I has NADPH-NAD and a NADH-NAD transhydrogenase activities. Each of these three activities has partially independent paths demonstrated by inhibitors specific for each. Complex I has proton pumping and ATP synthesis properties expressed in natural or artificial membranes. In summary, Complex I differs from the soluble, substrate dehydrogenases in having a very much more complex structure as well as residing within a membrane as a component of the electron transport system. The mitochondrial matrix enzymes, pyruvate dehydrogenase complex, α-ketoglutarate dehydrogenase complex, malate dehydrogenase and β-hydroxyacyl CoA dehydrogenase are known to be able to bind to Complex I [3]. The binding of these dehydrogenases to Complex I may provide an enzyme organization that enhances NADH oxidation in vivo.

Enzyme Associations and Substrate Channeling

In addition to the binding of some dehydrogenases to Complex I mentioned above, other mitochondrial enzymes can also form specific complexes with metabolically adjacent enzymes. Examples of association among mitochondrial enzymes include binary complexes [4], such as GDH-AAT, AAT-MDH, MDH-CS, GDH-MDH, PDC-CS, fumarase-MDH, fumarase-CS, CPS-OTC, KGDC-STK, AAT-CS, CS-thiolase, KGDC-ICD (NAD) [5], AAT-KGDC and CS-KGDC [6]; listed in order of published reports. Ternary enzyme complexes include: fumarase-MDH- with either CS or AAT, AAT-GDH-CPS, Complex I- KGDC- with either ICD (NAD) or STK [5] and MDH-KGDC with either AAT or CS [6]. Srere et al. reported that gentle sonic disruption could be used to obtain a mitochondrial preparation where Krebs cycle enzymes appeared to be organized in a large complex of proteins [1, 7]. He proposed the term metabolon for such a complex of sequential enzymes. The evidence for enzyme associations and their specificity with respect to enzymes of metabolically sequential reactions has promoted the idea that the associated enzymes can permit partial or complete channeling of the dissociable

intermediates between the enzymes; i.e., the product of the first enzyme may be utilized by the second enzyme before the intermediate diffuses from the molecular complex into the surrounding bulk phase. Substrate channeling provides a dynamic microcompartmentation of metabolic intermediates. The channeling can exist at least partially and for significant time periods, i.e., it can be perfect or leaky depending on the system and conditions. Such microcompartmention can provide significantly different catalytic properties than are possible in homogeneous phase catalysis [8]. Substrate channeling has been demonstrated in numerous systems [4] in all parts of the cells of microbes, plants, and animals. Recent data demonstrate substrate channeling in vivo of succinate and fumarate within the Krebs cycle [9] and of intermediates in cholesterogenesis [10]. Using permeabilized hepatocytes, Cheung et al. [11] demonstrated substrate channeling in the urea cycle involving enzymes from both cytoplasmic and mitochondrial compartments. Clegg and Jackson [12] present compelling evidence of substrate channeling in the glycolytic pathway using methods that rapidly remove the plasma membrane from cells. Other studies indicated the importance of enzyme organization in situ, most likely the consequence of substrate channeling. Robinson et al. and later Sumegi reported the considerable kinetic advantages of partially-, vs. completely- permeabilized mitochondria for Krebs cycle and fatty oxidation. They also found that the reaction velocities were much higher than would be expected from reasonable estimates of the unbound-metabolite concentrations. MacLennan and Tzagoloff [13] reported that maximal rates of oxidation and phosphorylation occurred with NAD concentrations no higher than those of the fixed components of the electron transport chain. This and the advantages of the structural organization in partially permeabilized mitochondria are hard to reconcile with a random arrangement of enzymes and intermediates.

A few of the published claims of substrate channeling have been challenged [14, 15]. Chock and Gutfreund [14] disputed the results of Srivastava and Bernhard for channeling of NADH from LDH to α-GPD [16] and of dihydroxyacetone-P from aldolase

to α–GPD [17]. The first of these systems has been thoroughly restudied in Srivastava's and independently in Spivey's laboratory. Their results [18] rigorously reaffirm the original interpretations of Srivastava and Bernhard, as do the earlier studies of others [19, 20, 21].

More pertinent to this thesis are the studies of NADH channeling from mitochondrial and cytoplasmic MDH, HAD, and GDH to Complex I. Efficient NADH channeling was found for each of these donor enzymes [22].

Methods to Study Substrate Channeling

Systems in vivo and in situ are important for demonstrating the existence, behavior, and biological relevance of substrate channeling, but studies in vitro are needed to clarify molecular mechanisms. Consider the sequential reactions A-> B-> P catalyzed by enzymes E1 and E2, respectively. Three different molecular mechanisms for channeling of B have been demonstrated in vitro.

A. By Direct Transfer Processes Involving

1. Stable E1-E2 complexes as e.g., in pyruvate dehydrogenase.
2. Transient E1-E2 complexes [16].

B. By Proximity Effects [8]

Each of these mechanisms shows significantly different experimental properties that are important for the elucidating and understanding their potential physiological consequences. In direct transfer mechanism, the intermediate is passed directly from the first enzyme (E1) to the second enzyme (E2) in the E1-E2 complex before the intermediate dissociates from the enzyme complex. Functional substrate channeling, however, can also be obtained when the intermediate dissociates from the first enzyme, if the local concentration of the second enzyme is sufficient to utilize the intermediate before it diffuses away. Four different, general methods have been used to demonstrate substrate channeling. These are: 1) the "enzyme buffering method" for transiently formed enzyme

complexes [16]; 2) effect of a competing reaction for the intermediate ("enzyme reaction trap") [23]; 3) measurements of the lag-time of the coupled reactions [24]; and 4) isotope dilution of intermediates. Method No. 1 was first used by Cori, et al. [19], and then by two other labs in the 1950's [20, 21]. These first demonstrations of substrate channeling were subsequently forgotten. Srivastava and Bernhard greatly expanded our understanding of this mechanism and demonstrated its prevalence [16]. The four general methods have different strengths and weaknesses depending on the mechanism of channeling and the system parameters. Method 1 is one of the few rigorous methods for studying the transient enzyme-enzyme mechanism. It is especially good for studies of NADH channeling between dehydrogenases. Recently, ^{31}P-NMR methods have also been successfully applied to the transient enzyme complex mechanism for channeling of ATP [25]. This method deserves serious consideration, but requires large quantities of both E1 and E2, and may be restricted to transfer of a few types of intermediates (ones with nuclei having large NMR chemical shifts). The other three methods listed above are generally applicable to stable enzyme complexes only.

Initially, attempts to demonstrate NADH channeling from substrate level dehydrogenases to Complex I were made with the competing reaction method. However, the fact that NAD and NADH unexpectedly reduced the binding of the dehydrogenases to Complex I and the lack of experimental success with preliminary measurements, persuaded us to switch to the enzyme buffering method. This has been used successfully in characterizing NADH channeling in these systems [22]. However, the enzyme buffering method has a few deficiencies. First, it requires larger amounts of donor enzymes than the other tests. In contrast, the competing reaction method requires nearly 10,000-fold less donor enzyme. Thus, if the competing reaction method can be adapted to the Complex I system, it would facilitate our planned studies with mutant forms of malate dehydrogenase. Purification of large amounts of these mutants is very time consuming, especially since purification properties vary with the type of mutant, as found by our colleague and

collaborator [26]. Secondly, the enzyme buffering method requires that the donor enzyme, E1 be completely free of activity of the acceptor enzyme E2. These purities are obtainable if the E2 activity in E1 preparations is due to extrinsic enzyme contamination. However, since the α-keto acid dehydrogenases have high intrinsic NADH oxidase activity, it is impractical to use a standard enzyme buffering test for NADH channeling with pyruvate dehydrogenase, α-ketoglutarate dehydrogenase complex, and branched chain keto acid dehydrogenases. A third motivation for adapting the competing reaction method to Complex I is to provide an independent test and characterization of NADH channeling in this Complex.

The study of substrate channeling is important if we are to understand the organization of enzymes in vivo, and its catalytic consequences.

Specific Aims

As a consequence of the above considerations, the objectives of this research are: 1) to adapt the competing reaction method to test for and characterize NADH channeling between mitochondrial malate dehydrogenase and Complex I within submitochondrial particles; 2) to characterize the binding of malate dehydrogenase to submitochondrial particles under higher ionic strengths than previously used; and 3) to measure the effect of other mitochondrial matrix enzymes simultaneously present on this binding.

CHAPTER II

MATERIALS AND METHODS

Materials

Porcine heart malate dehydrogenase (mitochondrial form in glycerol) and porcine heart lactate dehydrogenase were obtained from Boehringer Mannheim Corporation. NADH and NAD (from yeast, grade III), oxaloacetic acid (grade I), L-malate (disodium salt), pyruvate (sodium salt, type II), and polyethylene glycol-6000 (molecular weight of about 8,000) were obtained from Sigma Chemical Company. Potassium ferricyanide was obtained from Mallinckrodt Chemical Works. Antimycin A in absolute alcohol solution was made in our laboratory several years ago. Its commercial source is uncertain, but its activity was adequate as demonstrated in control experiments presented below.

Methods

Polyacrylamide Gel Electrophoresis and Densitometry

SDS-Polyacrylamide gel electrophoresis was done on a Hoefer Scientific Instruments vertical slab gel unit (model SE 250, "Mighty Small II") with 1.5 mm thick running gels of 11.5% gel monomer and 2.7% cross-linker and the Laemmli system as described in the manufacturer's manual. For the mitochondrial matrix extract of enzymes, 10 μg of total SDS denatured protein were loaded into each lane. After electrophoresis, the gels were stained overnight with Coomassie Blue as specified in the manual and destained with a solution of 40% methanol, 10% acetic acid. Low range molecular weight markers (Bio-Rad) were added to one of the lanes. The wet gels were placed in clear plastic bags

and scanned with a Xerox flat bed optical scanner, model 730GS, interfaced to a Macintosh II computer. The resulting images were analyzed by the "GelReader" program, version 1.2b, obtained from the National Center for Supercomputing Applications (NCSA). This public domain program was obtained from NCSA by electronic mail from the address ZAPHOD.NCSA.UIUC.EDU and password, "Anonymous". This transfer was accomplished by way of our University's Computer Center using their VAX and FTP program. Using this program, we calculated molecular weights of each peak and the individual and summed areas of each peak. The peak of 35 kDa was assumed to represent malate dehydrogenase subunits.

Enzyme Preparations, Assays and Protein Concentrations

Porcine heart mitochondrial malate dehydrogenase (in glycerol) was used as a stock enzyme solution without further modification. The specific activity of mitochondrial malate dehydrogenases was determined in 20 mM potassium phosphate buffer, pH 7.5, containing 0.2 mM NADH, 0.15 mM oxaloacetate and 1 mg/ml BSA by following the decrease of absorbance at 340 nm and 25 ^{o}C. A molar absorptivity of 6.22 mM^{-1} cm^{-1} was used for NADH.

Porcine heart lactate dehydrogenase (ammonium sulfate suspension) was centrifuged in a Brinkmann 5415 microcentrifuge at top speed for 20 minutes. The supernatant was discarded and the enzyme pellet was dissolved in 20 mM potassium phosphate buffer, pH 7.5. The specific activity of lactate dehydrogenase was determined in 20 mM potassium phosphate buffer, pH 7.5, containing 0.63 mM pyruvate and 0.2 mM NADH by following the decrease of absorbance at 340 nm and 25 ^{o}C.

All protein concentrations were determined by the Lowry method [27].

Beef Heart Submitochondrial Particles

Submitochondrial particles were prepared by the method of H. James Harmon [28]

with minor modifications and the following centrifugation details. All procedures were done with materials kept at 0 - 4 °C. Several beef hearts were collected from freshly slaughtered cattle and placed in ice. Ventricles from about 7 kg of beef heart were trimmed and ground. Two liters of 0.25 M sucrose containing 30 mM Tris base was added to each 800 ml mince followed by blending for 45 seconds at low speed. The mixture was adjusted to pH 7.0 - 7.2 with 2.0 M Tris base and centrifuged at 527 x g for 20 minutes in a Beckman J-6 centrifuge. The supernatant was filtered through 8 layers of cheesecloth and centrifuged at 27,000 x g for 15 minutes in a Sorval RC-5B centrifuge. The pellet was washed by homogenizing it in a small volume, resuspending in 1,300 ml of 0.25 M sucrose and centrifuging at 27,000 x g for 15 minutes. The pellet wash was repeated once. Both heavy and light mitochondria were sedimented and collected. Protein concentrations were determined by the biuret method using positive displacement pipettes for the mitochondrial suspension.

For preparation of the submitochondrial particles the mitochondrial suspension was diluted to a protein concentration of 30 mg/ml with 10 mM potassium phosphate, pH 7.4 , containing 0.25 M sucrose, and sonicated for 30 seconds in a 125 ml stainless steel beaker at 40% power output using a Heat System Model W-220 F sonicator with 1/2 inch diameter tip. The sonicated mixture was centrifuged at 23,500 x g for 10 minutes. Then 10 ml of 0.25 M sucrose and 0.15 M potassium chloride were added to the supernatant following by centrifuging at 44,000 x g for 30 minutes. The pellet was washed by homogenizing and resuspending in 10 ml of 0.25 M sucrose, 0.15 M potassium chloride and centrifuged at 44,000 x g for 25 minutes. This wash was repeated one more time. The pellet of submitochondrial particles, suspended in 0.25 M sucrose, was stable for several months when kept at -20 °C. The extent to which these inner membrane vesicle particles had the desired inverted membrane orientation (inside surface on the outside) was determined by measuring the NADH oxidase activity spectroscopically at 340 nm with and without

cytochrome c in 20 mM potassium phosphate buffer, pH 7.5 containing 0.1 mM NADH [28]. By this criterion, 80% of the submitochondrial particles were inverted.

The specific activity of NADH dehydrogenase in submitochondrial particles was determined spectroscopically at 410 nm with ferricyanide as the electron acceptor. Assay conditions were 0.1 mM NADH, 1.25 mM potassium ferricyanide, and 0.01 μg/ml antimycin A in 20 mM potassium phosphate buffer, pH 7.5 at 25 °C. A molar absorptivity of 1.0 mM^{-1} cm^{-1} was used for ferricyanide.

Extract of Mitochondrial Matrix Enzymes

To obtain a mixture of all mitochondrial matrix enzymes and retain relative concentrations representative of the native mitochondria, 6 ml of 2 mM HEPES buffer, pH 7.5, containing 0.25 M sucrose was added to 30 ml of mitochondria suspension (30 mg protein/ml initially; i.e., 900 mg total protein). This suspension was sonicated for five periods of five seconds each, with brief non-sonicating intervals in between. The Heat Systems Model W-220 F sonicator was used at 40% power output with a 1/2 inch diameter tip. The mixture was centrifuged at 44,000 x g for 30 minutes. Then 0.1 ml of 3 M potassium chloride was added to 4 ml of supernatant. The pellet was suspended in 5 ml of 2 mM HEPES buffer, pH 7.5, containing 0.2 M sucrose and 0.15 M potassium chloride and centrifuged at 44,000 x g for 30 minutes. The specific activity of malate dehydrogenase in the supernatant was determined as described above. The weight fraction of protein in the mitochondrial extract that is malate dehydrogenase (MDH) can be calculated by two independent measurements that give similar results, 6 and 7%, respectively. First, a specific activity of 60 U-MDH/mg matrix protein was measured, where a unit U of activity is defined as the amount of enzyme producing 1 μmol product/min in the standard assay. Thus, assuming a typical specific activity of the pure MDH of 1000 U/mg-MDH, gives 6% weight fraction. Secondly the mitochondrial extract

was subjected to SDS-PAGE and densitometric analysis as described above. The area of the assumed MDH peak was 7% of the combined areas from all peaks.

Preparation of Substrate Solutions

NADH, pyruvate, malate and potassium ferricyanide were dissolved in 20 mM potassium phosphate buffer, pH 7.5. NAD solutions (40 mM) were freshly prepared in water. This acid solution was incubated for 30 minutes at room temperature to destroy trace amounts of NADH [29]. Then it was diluted with an equal volume of 40 mM potassium phosphate buffer, pH 7.5 giving a final 20 mM NAD solution in 20 mM potassium phosphate buffer. The excess protons were neutralized by the addition of 1.25 µl of 1.0 N sodium hydroxide per mg of NAD. Oxaloacetic acid was dissolved in 20 mM potassium phosphate buffer, pH 7.5, and neutralized by the addition of 15 µl of 1.0 N sodium hydroxide per mg of oxaloacetate.

Substrate Channeling Measurement

A competing reaction method was used to measure the NADH channeling from malate dehydrogenase to submitochondrial particles. The following reaction is catalyzed by malate dehydrogenase:

$$\text{Malate} + \text{NAD} \xrightarrow{\text{MDH}} \text{Oxaloacetate} + \text{NADH}$$

The NADH produced by this reaction can be reoxidized by NADH-ubiquinone oxidoreductase (Complex I) in submitochondrial particles as follows:

$$Fe(CN)_6^{3-} + \text{NADH} \xrightarrow{\text{SMP}} Fe(CN)_6^{4-} + \text{NAD}$$

These two coupled reactions were started by the addition of malate dehydrogenase to a mixture of malate, NAD, $Fe(CN)_6^{3-}$, antimycin A, and SMP in 20 mM potassium phosphate buffer, pH 7.5. The rate of the coupled reaction was determined by measuring

the reduction of ferricyanide as the decrease of absorbance at 410 mM. To test the substrate channeling of NADH, an excess of lactate dehydrogenase and its substrate, pyruvate, were used to trap any free-NADH which was not channeled to Complex I. Thus, unless substrate channeling occurs, the reduction of ferricyanide should be prevented by the competing reaction catalyzed by lactate dehydrogenase.

Binding Measurements

Submitochondrial particles and malate dehydrogenase (total of 100 μl) were incubated in 20 mM potassium phosphate buffer, pH 7.5, on ice for 30 minutes followed by centrifugation in a Brinkmann 5415 microcentrifuge at top speed for 30 minutes. Submitochondrial particles and bound malate dehydrogenase were sedimented. Independent experiments with SMP alone or MDH alone established that all of the SMP but none of MDH are pelleted under these conditions. The pellet was resuspended in 20 mM potassium phosphate buffer, pH 7.5. The activity of malate dehydrogenase in both supernatant and pellet was determined at 25 ^{o}C. Trace ^{14}C-labelled malate dehydrogenase, prepared as previously described for protein labelling [30], was also used in some binding experiments. After incubation and centrifugation the amounts of malate dehydrogenase in both the supernatant and pellet phases were determined by measurements with a scintillation counter.

CHAPTER III

RESULTS AND DISCUSSION

Existence of NADH Channeling

Control Experiments

Control measurements are shown in Figure 2. The absorbance at 340 nm was measured starting with 20 mM potassium phosphate buffer, pH 7.5, containing 10 mM malate and 2 mM NAD. Sequential additions as indicated by the arrows were made and responses observed as follows. Final reaction concentrations are given in parentheses. Addition of malate dehydrogenase (2 μg/ml) caused an increase in NADH concentration and absorbance. Subsequent addition of submitochondrial particles (SMP) (240 μg/ml) caused a rapid depletion of the NADH due to the NADH oxidase activity of the electron transport system on the SMP. This NADH oxidation was completely inhibited by the addition of antimycin A (0.03 μg/ml), i.e., the rate of NADH production from the malate dehydrogenase reaction was equal to that before addition of SMP. Addition of excess lactate dehydrogenase (0.2 mg/ml) and pyruvate (10 mM), however, consumed the NADH rapidly. In summary, these results indicate that : 1) the NADH generated by malate dehydrogenase could be oxidized to NAD by "NADH oxidase" in submitochondrial particles; 2) antimycin A inhibited the reoxidation of NADH by "NADH oxidase" in submitochondrial particles; 3) the reaction catalyzed by lactate dehydrogenase was very effective for trapping free-NADH in the bulk phase under our experimental condition. These reactions are as follows:

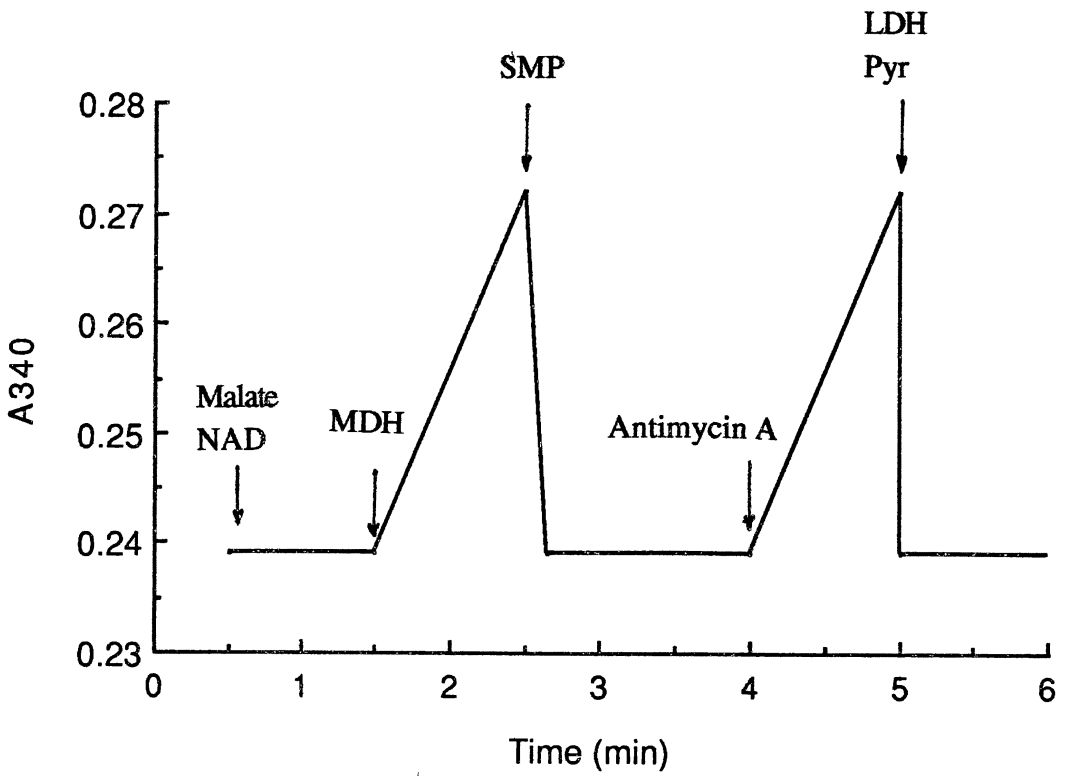
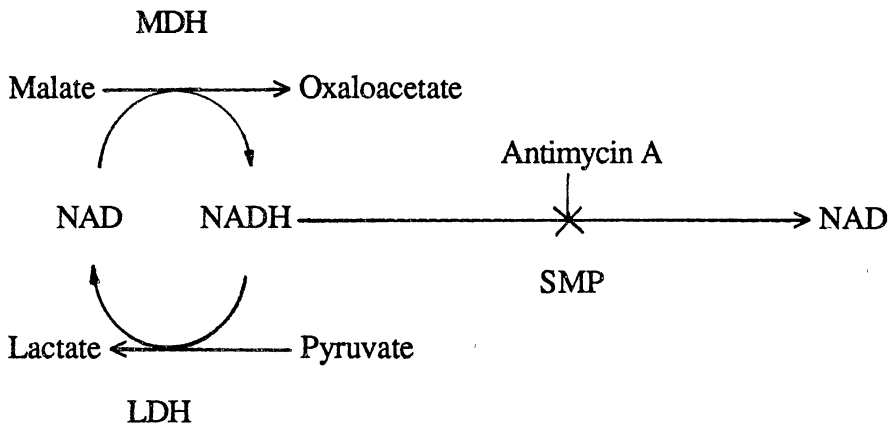


Figure 2. Absorbance Curve of Control Experiment



NADH Channeling Test

To test the substrate channeling of NADH from mitochondrial malate dehydrogenase to submitochondrial particles, we followed the ferricyanide reduction by Complex I using NADH (generated by the MDH reaction) as electron donor. Malate dehydrogenase and submitochondrial particles were first incubated at different weight ratios in 20 mM potassium phosphate buffer, pH 7.5, on ice for 20 minutes. This incubation was to promote MDH binding to Complex I. The $Fe(CN)_6^{3-}$ reaction was started by the addition of the incubated mixture to the assay solution containing 10 mM malate, 2 mM NAD, 1.25 mM $Fe(CN)_6^{3-}$, and 0.3 μg/ml antimycin A in 20 mM potassium phosphate buffer at pH 7.5 and 25 ^{0}C When present, LDH was at 0.2 mg/ml with 0.1 mM pyruvate. The NADH channeling was observed when 2 μg/ml MDH and 240 μg/ml SMP were incubated and transferred to the reaction mixture giving final concentration of 2 μg/ml MDH and 240 μg/ml SMP; i.e., a weight ratio of 1:120 for MDH:SMP. The reaction rate of $Fe(CN)_6^{3-}$ reduction at 410 nm was 0.072±0.003 absorbance units per minute (3 determinations) in the absence of lactate dehydrogenase and its substrate (Figure 3A). In the presence of excess lactate dehydrogenase (0.2 mg/ml) and 0.1 mM pyruvate, the absorbance change at 340 nm per minute decreased to 0.026±0.002 (3 determinations), (Figure 3B). When the amount of lactate dehydrogenase was doubled, the same trapping

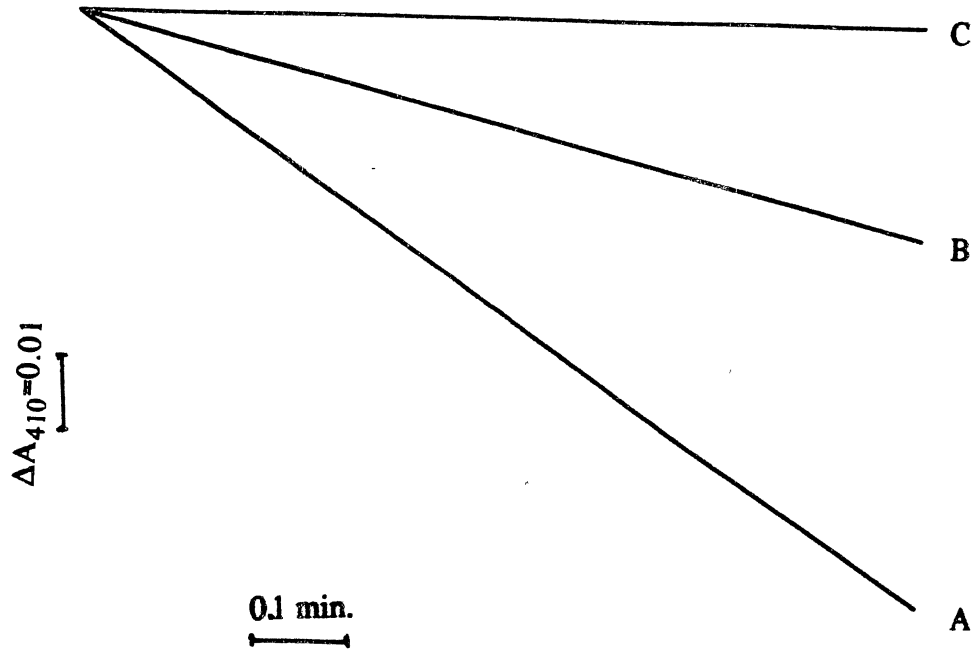


Figure 3. Absorbance Curves of Substrate Channeling Tests.
Curve A: 2 μg MDH and 240 μg SMP were incubated in 50 μl of potassium phosphate buffer, pH 7.5, on ice for 30 minutes. The absorbance change at 410 nm per minute was measured by the addition of 20 μl of the incubated enzyme mixture into 0.98 ml of assay solution containing 10 mM malate, 2 mM NAD, 1.25 mM $Fe(CN)_6^{3-}$ and 0.3 μg/ml antimycin A in 20 mM potassium phosphate buffer, pH 7.5, at 25 °C. Curve B: The same condition as that of Curve A but with 0.2 mg/ml lactate dehydrogenase and 0.1 mM pyruvate also present in the assay solution. Curve C: The same condition as that of Curve B was used except 24 instead of 240 μg SMP was incubated with 2 μg MDH.

percentage was obtained. When SMP concentration was decreased 10-fold, the coupled reactions had the same rate in the absence of LDH (Figure 4), but complete trapping of NADH occurred with LDH present (Figure 3C). The results of Figure 4 demonstrate that the MDH reaction remains rate limiting even with the 10-fold dilution of SMP to 24 μg/ml SMP protein. This is consistent with the fact that a much smaller fraction of the MDH can be bound to SMP receptors with the lower SMP concentrations.

Thus, with these experimental conditions, about 36% of the NADH produced by malate dehydrogenase is passed directly to submitochondrial particles without first dissociating into the bulk phase. The Complex I within the submitochondrial particles oxidized the channeled NADH and transferred the electrons from NADH to $Fe(CN)_6^{3-}$. About 64% of the NADH produced by malate dehydrogenase dissociated into the bulk phase and was trapped by lactate dehydrogenase. These results indicate the existence of NADH channeling from mitochondrial malate dehydrogenase to submitochondrial particles.

Results shown in Figure 4 clearly demonstrate that with 2 μg/ml MDH and 24 μg/ml or 240 μg/ml SMP, the MDH reaction is rate limiting.

Polyethylene glycol enhances the association of MDH with SMP under many conditions [31, 32]. Therefore, the effect of PEG on NADH channeling was tested. The malate dehydrogenase and submitochondrial particles (at the same concentrations as in the previous experiment) were incubated with 10% (w/v) polyethylene glycol on ice for 20 minutes. Then an aliquot of incubated mixture was added to the reaction vessel. The absorbance change at 410 nm in the first minute was about twice that without polyethylene glycol, then decreased to the same rate as that without polyethylene glycol. According to "excluded volume theory" [33], the proteins are sterically excluded from the solvent phase occupied by PEG and are thus concentrated. This increased concentration enhances the association equilibria. The decrease in observed reaction rate with time is probably due to the dissociation of MDH from Complex I after the MDH-Complex I mixture is added to and diluted by the assay solution. In the presence of polyethylene glycol, lactate

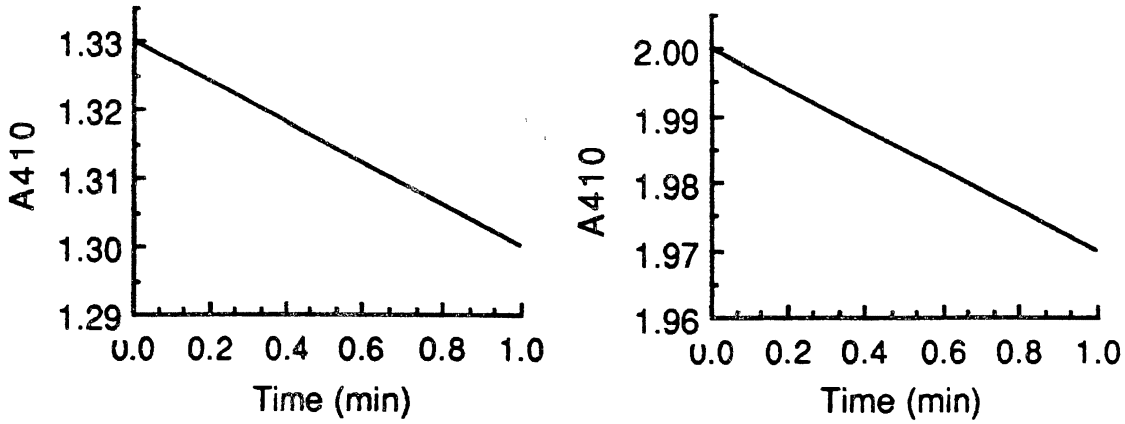


Figure 4. Identification of the MDH Reaction as Rate Limiting Step in the Coupled Reactions Reducing Ferricyanide. The absorbance change at 410 nm was measured by the addition of 2 μg/ml MDH and 24 μg/ml SMP (for left figure) or 240 μg/ml SMP (for right figure) into assay solution containing 10 mM malate, 2 mM NAD, 1.25 mM $K_3Fe(CN)_6$ and 0.3 μg/ml antimycin A in 20 mM potassium phosphate buffer, pH 7.5, at 25 °C. Equal rates are shown for both concentrations of SMP.

dehydrogenase trapped about 50% of the NADH generated by malate dehydrogenase. This result shows that polyethylene glycol enhances NADH channeling from malate dehydrogenase to submitochondrial particles.

The Binding of malate dehydrogenase to submitochondrial particles

Previous binding data of our laboratory have established that both purified Complex I and SMP can bind several dehydrogenases very extensively at low ionic strength. Fahien et al. [31] also report extensive binding of aspartate aminotransferase and malate dehydrogenase at low ionic strengths. However, binding of dehydrogenases at these low ionic strengths is generally in great excess of a 1:1 stoichiometry with Complex I, especially with the SMP. This indicates substantial binding at sites different from Complex I protein subunits. Thus it is important to measure the binding at higher ionic strengths to see if more specific and stoichiometric dehydrogenase binding can be obtained.

Malate dehydrogenase of varying concentrations (from 0 to 50 μg/ml) were mixed with submitochondrial particles (1.0 mg/ml protein) in 20 mM potassium phosphate buffer, pH 7.5, in a final volume of 0.1 ml. The mixtures were incubated on ice for 30 minutes and then centrifuged in a Brinkmann 5415 microcentrifuge at top speed and 4 ^{o}C for 30 minutes. Two methods were used to measure the amount of bound malate dehydrogenase to submitochondrial particles in the pellets and unbound malate dehydrogenase in the supernatants. One was a malate dehydrogenase activity assay and the other was radioactivity measurement when ^{14}C labeled malate dehydrogenase was used. The binding was found to be saturable. Submitochondrial particles containing 1 mg of protein can bind about 9 μg of malate dehydrogenase, a weight ratio of 1:120 (MDH to SMP protein), shown in Figure 5. The same experiment was repeated with twice as much SMP concentration (2 mg/ml). Practically twice as much malate dehydrogenase was pelleted giving the same binding ratio of MDH to SMP protein. The binding results indicate that malate dehydrogenase and submitochondrial particles can form stable complexes. Based

on the ratio of 0.06-0.07 nmol Complex I/mg SMP protein [34], 4.6 μg MDH/mg SMP protein corresponds to a 1:1 molar ratio (MDH:Complex I). Therefore the binding molar ratio of MDH to Complex I is about 2:1.

The binding of MDH from beef heart mitochondrial matrix extract to submitochondrial particles was measured using the same conditions as in the binding experiments with porcine heart malate dehydrogenase. First the mitochondrial matrix extract in varying concentrations was incubated with submitochondrial particles in 20 mM potassium phosphate buffer, pH 7.5, on ice for 30 minutes. Then the mixture was centrifuged in a Brinkmann 5415 microcentrifuge at top speed and 4 ^{o}C for 30 minutes. The malate dehydrogenase activity of both supernatants and pellets was determined. Binding was found to be saturable at about 1.3 U-MDH/mg SMP protein. Assuming a specific activity of between 850-1000 U-MDH/mg for pure MDH, gives a weight binding ratio of 1.3-1.5 μg MDH/mg SMP protein. This in turn is about 0.3 mol MDH/mol Complex I. The reason the binding ratio υ was less than with MDH alone (9) is probably due to the aspartate aminotransferase and other enzymes in the mitochondrial matrix extracts. Fahien et al [31] have recently demonstrated that aspartate aminotransferase reduces binding of MDH to submitochondrial particles.

Figure 5. Binding of MDH to SMP. The MDH in increasing concentrations was incubated with 1.0 mg/ml protein concentration of SMP in 20 mM potassium phosphate buffer, pH 7.5, on ice for 30 minutes. The incubated mixture was centrifuged in Brinkmann microcentrifuge at top speed for 30 minutes. Bound MDH was determined as described under "Materials and Methods". The values were obtained from four experiments.

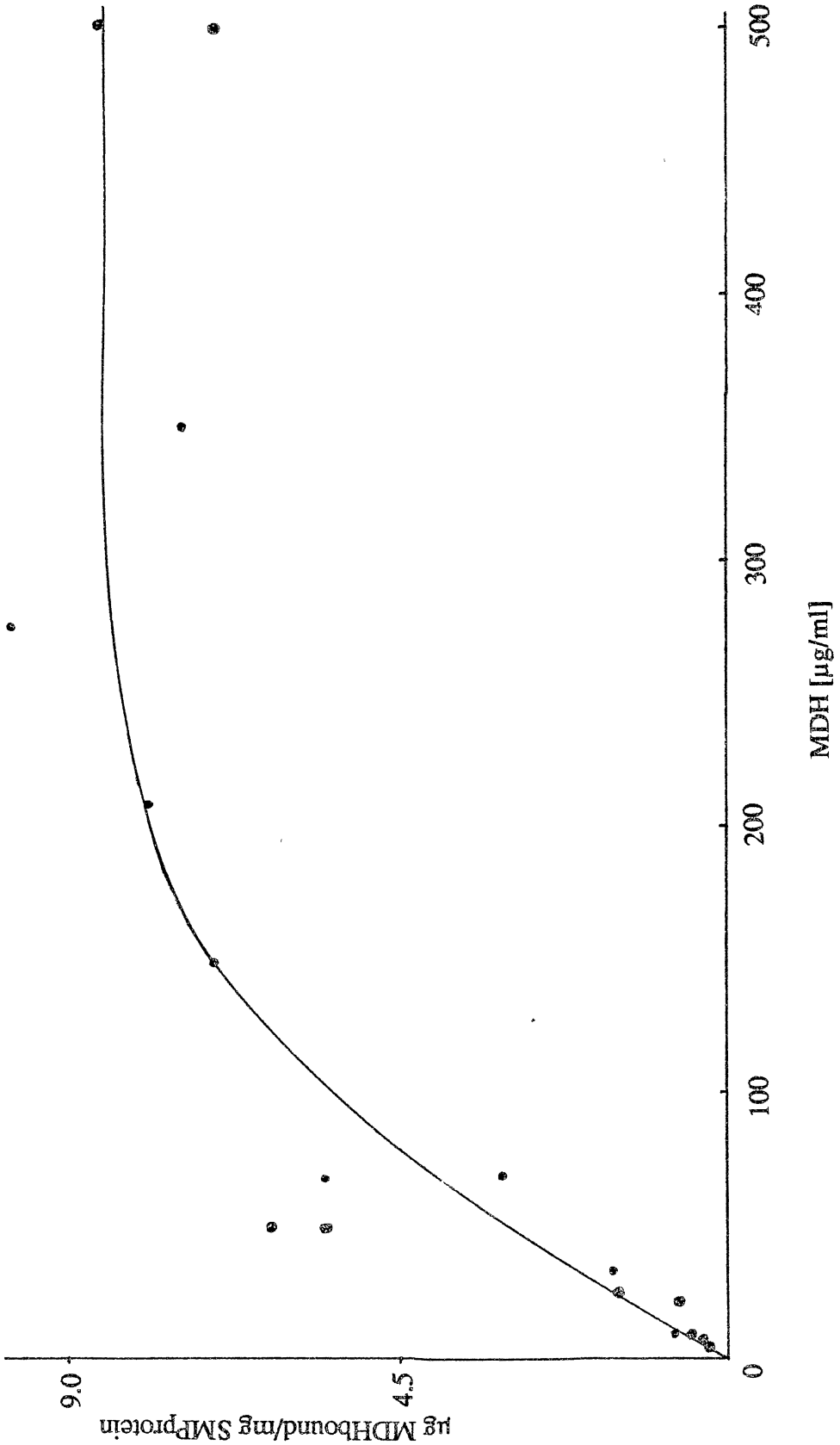
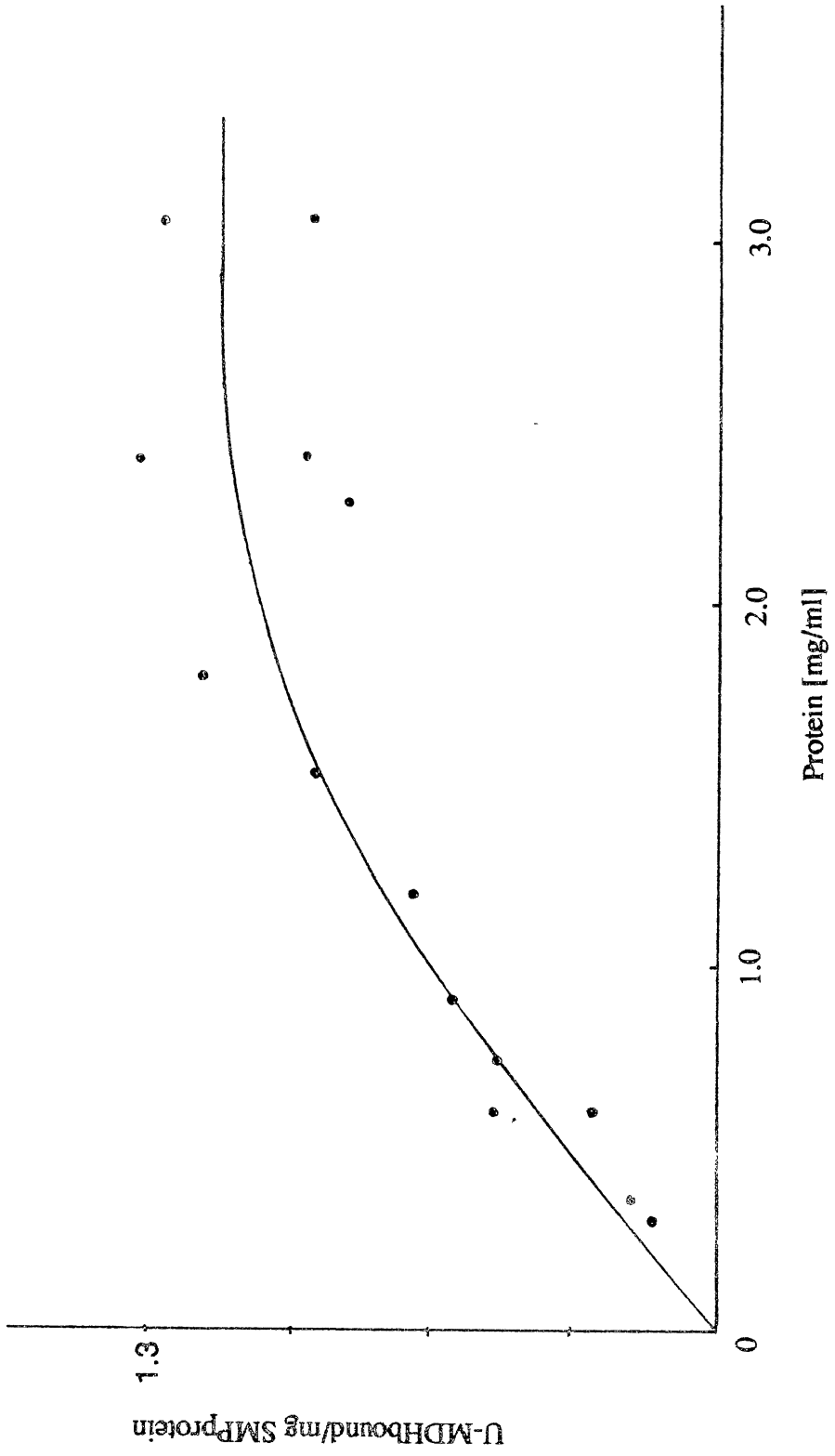

µg MDHbound/mg SMPprotein
9.0
4.5
0
100
200
300
400
500
MDH [µg/ml]

Figure 6. Binding of MDH in Mitochondrial Matrix Extract to SMP. The mitochondrial matrix extract in increasing concentrations was incubated with 1.0 mg/ml SMP in 20 potassium phosphate buffer, pH 7.5, on ice for 30 minutes. The incubated mixture was centrifuged in a Brinkmann 5415 microcentrifuge at top speed for 30 minutes. Bound MDH was determined by the measurement of MDH activity at 25 ^{o}C.


U-MDHbound/mg SMPprotein
1.3
0
1.0
2.0
3.0
Protein [mg/ml]

CHAPTER IV

CONCLUSION

Substrate channeling of NADH from porcine heart malate dehydrogenase to beef heart mitochondrial particles was observed with 2 mM NAD, 10 mM malate, 2 μg/ml malate dehydrogenase, 1.25 mM $Fe(CN)_6^{3-}$, 0.3 μg/ml antimycin A, 240 μg/ml protein of submitochondrial particles in 20 mM potassium phosphate buffer, pH 7.5. The weight ratio of malate dehydrogenase to submitochondrial particles was 1:120, corresponding to a molar ratio of about 2:1 (MDH:SMP). About 36% of the NADH generated by the malate dehydrogenase reaction was directly transferred to submitochondrial particles without dissociating into bulk phase. About 64% of the NADH dissociated into the bulk phase and was effectively trapped by the lactate dehydrogenase reaction.

The binding of malate dehydrogenase to submitochondrial particles in the same buffer was saturable at a binding ratio of 9 μg of bound malate dehydrogenase per mg protein of submitochondrial particles, corresponding to a molar ratio of 2:1 (MDH:SMP), in 20 mM potassium phosphate buffer at pH 7.5 and 4 °C. Polyethylene glycol enhanced the binding of MDH to SMP, as well as NADH channeling.

The malate dehydrogenase in mitochondrial matrix extract binds to submitochondrial particles with saturation at about 1.3 μg MDH/ mg SMP protein, which corresponds to a 0.3:1 molar ratio. The aspartate aminotransferase in this extract may be interfering with MDH binding as described by Fahien et al.[31] although several other matrix enzymes may also be responsible.

Submitochondrial particles are a good test model for substrate channeling studies since they provide a native and complete set of enzyme binding sites as well as Complex I within its natural membrane environment.

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